RESEARCH PAPER

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Differential expression of miR200a-3p and miR21 in grade II–III and grade IV gliomas

Evidence that miR200a-3p is regulated by O^6-methylguanine methyltransferase and promotes temozolomide responsiveness

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Keywords: glioblastoma, microRNA, miR-200a-3p, miR21, temozolomide, O^6-methylguanine methyltransferase, chemoresistance

Abbreviations: EMT, epithelial–mesenchymental transition; FBS, bovine serum; GBM, glioblastoma; GFAP, glial fibrillary acidic protein; LG, lower grade; MGMT, O^6-methylguanine methyltransferase; miRNA, microRNA; MMLV, moloney murine leukemia virus reverse transcriptase; MTT, thiazolyl blue tetrazolium blue; O^6-BG, O^6-benzylguanine; PI3K, phosphoinositide-3-kinase; PTEN, phosphatase and tensin homolog; TMZ, temozolomide; RT-PCR, reverse transcription polymerase chain reaction; STUPP, radiotherapy plus concomitant and adjuvant temozolomide; WHO, World Health Organization

Glioblastoma multiforme (GBM) is the most common primary brain tumor and is among the deadliest of human cancers. Dysregulation of microRNAs (miRNAs) expression is an important step in tumor progression as miRNAs can act as tumor suppressors or oncogenes and may affect cell sensitivity to chemotherapy. Whereas the oncogenic miR21 has been shown to be overexpressed in gliomas, the expression and function of the tumor-supressor miR200a in GBMs remains unknown. In this study, we show that miR21 is upregulated in grade IV (GBMs) vs. grade II–III (LGs) gliomas, confirming that miR21 expression level is correlated with tumor grade, and that it may be considered as a marker of tumor progression. Conversely, miR200a is demonstrated for the first time to be downregulated in GBMs compared with LGs, and overexpression of miR200a in GBM cells is shown to promote TMZ-sensitivity. Interestingly, miR200a but not miR21 expression level is significantly higher in TMZ-responsive vs. -unresponsive tumoral glial cells in primary culture. Furthermore, miR200a appears negatively correlated with the expression of the DNA repair enzyme O^6-methylguanine methyltransferase (MGMT), and the inhibition of MGMT activity results in an increase of miR200a expression in GBM cells. Taken together, these data strongly suggest that miR200a is likely to act as a crucial antitumoral factor regarding glioma progression. Interplay between miR200a and MGMT should be considered as potential mechanism involved in therapeutic response.

Introduction

Glioblastoma multiforme (GBM) is the most common primary brain tumor and among the deadliest of human cancers. Despite advances in diagnosis and treatment, mean survival time does not exceed 2 y. These tumors typically display a very high proliferative rate with widespread microvascular development and areas of focal necrosis.[1] The current standard therapy for GBM includes surgery with maximum feasible resection, radiotherapy, and treatment with the monofunctional alkylating agent temozolomide (TMZ).[2,3] Although TMZ has improved overall and progression-free survival, a majority of patients still experience disease progression within 1 y. Indeed, GBMs can present innate resistance or develop acquired resistance during TMZ treatment.[4-6]

Resistance of GBMs to TMZ is reported to be mainly, but not exclusively, dependent on high levels of the DNA repair enzyme, O^6-methylguanine methyltransferase (MGMT), which can reverse the methylation damage induced by alkylating agents.[7-9] Although a number of studies have shown that a deficiency of MGMT can augment the sensitivity of GBMs to alkylating agents such as TMZ, a panel of tumors with low levels of MGMT are nevertheless chemoresistant.[10,11] This suggests that additional mechanisms are involved in tumor resistance to

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chemotherapy.[12] Thus, resistance of GBM cells to TMZ includes various events, such as loss of the phosphatase and tensin homolog (PTEN), leading to the activation of the phosphoinositide-3-kinase (PI3K)/Akt pathway, deficiency in the DNA mismatch repair system, mutations in the pro-apoptotic p53 protein, overexpression of the anti-apoptotic protein Bcl-2, or the selection of less-differentiated pre-existing resistant cells in the parental tumor.[13-19]

Dysregulation of the expression of microRNAs (miRNAs) is emerging as an important step in early processes during tumorigenesis as well as disease progression/metastasis in various malignancies including GBMs.[20-22] The miRNAs function mainly through their ability to bind to the 3′ untranslated regions of mRNAs, leading to their degradation or suppression of their translation.[23] MiRNAs can act as tumor suppressors or oncogenes depending upon the nature of the targeted genes.[24] MiRNA profiles have been used successfully to classify tumors and predict response to therapies.[25,26] These analyses have identified a number of miRNAs consistently altered in gliomas, and one predominant miRNA to arise from these studies is miR21.[27] Thus, the miRNA expression studies demonstrated that miR21 is overexpressed in gliomas and glioma cell lines relative to normal tissue.[28] However, the association of miR21 with tumor grade is less consistent. While some studies indicate that miR21 is upregulated in both low- and high-grade gliomas, other suggest that it is associated with progression and upregulated more frequently in high-grade tumors.[28,29] Whereas the correlation of miR21 with clinical outcome in gliomas is little documented, miR21 expression level has been correlated with overall and disease-free survival and suggested to be a biomarker for chemoresistance in other types of cancer including leukemia, pancreatic and lung cancers.[30-33]

Besides oncogenic miRNAs such as miR21, a number of miRNAs have been described to elicit tumor suppressive role and to display downregulation during tumor development.[24] This is the case for the miR200 family, composed of five members: miR-200a, -200b, -200c, -141, and -429. Members of the mir200 family have been shown to be major regulators of the EMT (epithelial–mesenchymental transition) process, primarily by targeting EMT-driving transcription factors ZEB1 and ZEB2 (SIP1), which in turn transcriptionally repress miR200 family members in a double negative feedback loop.[34,35] A number of studies indicate that members of the miR200 family are downregulated in a variety of human cancers such as breast, ovarian and lung cancers.[36-38] Moreover, a role for miR200 family in drug resistance has been reported in several cancers. Thus, gemcitabin-resistant pancreatic cancer is associated with decreased miR200 expression.[39] Similarly, expression level of miR200a is correlated with resistance of ovarian and bladder cancers to paclitaxel and anti-EGFR therapy, respectively.[36,40] However, contradictory data regarding these microRNAs have been reported, since other studies describe overexpression of miR200 family members in cancers such as melanoma, ovarian and colorectal cancers.[41-44] Contrary to miR21, the role of miR200 in brain tumors is little documented. Whereas Saydam et al.[45] have shown that downregulation of miR200a in meningiomas increased tumor growth through the activation of the wnt/β-catenin signaling pathway,

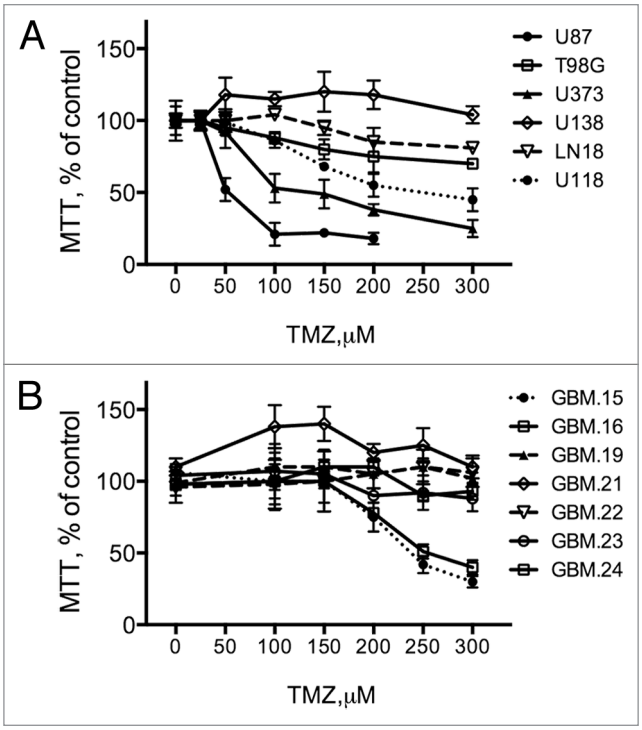

Figure 1. Effect of temozolomide (TMZ) on the proliferation of tumoral glial cell lines (**A**) and glioblastoma-derived cell primary cultures (**B**). Cells were treated for 3 d in the presence of the indicated concentrations of TMZ. Cell proliferation was then assessed by MTT metabolism. Values are expressed as % of untreated control cells. Data are the mean ± SD of 3 separate experiments.

the expression and function of miR200 in glioblastomas have not been investigated and remain unknown.

In order to clarify the functional role of miR21 and more specifically miR200a in glioblastomas, the expression level of both miRNAs was first analyzed and compared in grade IV (GBMs) and grade II–III (LGs) glioma samples. We show that miR21 is upregulated in GBMs when compared with LGs, confirming that miR21 expression level is correlated with tumor grade, and that it may be considered as a marker of tumor progression. Conversely, miR200a is demonstrated for the first time to be downregulated in GBMs compared with lower grade gliomas. Interestingly, downregulation of miR200a in GBMs is correlated with MGMT expression level and TMZ-resistance, strongly suggesting that this miRNA is likely a crucial factor when considering glioma progression and therapeutic response.

Results

Effect of TMZ on glioma cell proliferation

To evaluate the antitumor effect of TMZ in malignant glioma cells, several glial tumoral cell lines were treated with increased doses of TMZ for 72 h. Cell viability was then assessed by MTT metabolism. As shown in **Figure 1A**, variable cell growth inhibitory effects of TMZ were observed, depending on the tumor cell lines examined. Whereas U138, T98G, and LN18 cells were

Table 1. Characterization of the tumoral glial cell lines used in this study

Cell lines	MGMT/18s	MGMT protein	MGMT methylation	miR200a/U6	TMZ IC_{50}, μM
U87	$1.0\ 10^{-6} \pm 2.010^{-6}$ [1]*	nd	M	$2.0\ 10^{-3} \pm 1.1\ 10^{-3}$	50
U373	$5.1\ 10^{-6} \pm 2.8\ 10^{-6}$ [5.1]	nd	M	$1.1\ 10^{-3} \pm 2.2\ 10^{-3}$	110
T98G	$1.1\ 10^{-3} \pm 0.9\ 10^{-3}$ [1.1 10^{3}]	+	M	$1.0\ 10^{-4} \pm 0.07\ 10^{-4}$	>300
U118	$2.0\ 10^{-3} \pm 1.1\ 10^{-3}$ [2 10^{3}]	+	UM	$1.0\ 10^{-3} \pm 0.6\ 10^{-4}$	220
U138	$2.4\ 10^{-2} \pm 0.6\ 10^{-2}$ [2.4 10^{4}]	++	M+UM	$1.2\ 10^{-4} \pm 1.1\ 10^{-4}$	>300
LN18	$3.1\ 10^{-2} \pm 0.3\ 10^{-2}$ [3.1 10^{4}]	++	UM	$3.1\ 10^{-4} \pm 1.9\ 10^{-4}$	>300

*in brackets, relative expression level of MGMT, taking MGMT level in U87 cells as 1; nd, no detectable; M, methylated MGMT; UM, unmethylated MGMT.

Table 2. Characterization of the glioblastoma-derived cell primary cultures used in this study

Primary cultures	MGMT/18s	MGMT protein	MGMT methylation	miR200a/U6	TMZ IC_{50}, μM
GBM.4	0.21 ± 0.08 [2 10^{5}] *	+	M	$1.1\ 10^{-2} \pm 1.6\ 10^{-2}$	>300
GBM.5	$7.0\ 10^{-3} \pm 0.8\ 10^{-3}$ [5 10^{3}]	+	M	0.11 ± 0.51	>300
GBM.7	$2.5\ 10^{-3} \pm 0.7\ 10^{-3}$ [2.5 10^{3}]	+	UM	0.53	>300
GBM.8	$5.0\ 10^{-3} \pm 1.1\ 10^{-3}$ [5 10^{3}]	+/–	M		>300
GBM.9	$2.1\ 10^{-4} \pm 1.0\ 10^{-4}$ [2 10^{2}]	nd	M	1.12 ± 0.22	250
GBM.10	$0.9\ 10^{-4} \pm 0.3\ 10^{-4}$ [0.9 10^{2}]	nd	M		250
GBM.11	$2.3\ 10^{-4} \pm 0.9\ 10^{-4}$ [2.3 10^{2}]	nd	M	0.12	220
GBM.12	0.83 ± 0.03 [8 10^{5}]	++	UM	$3.0\ 10^{-3} \pm 0.6\ 10^{-3}$	>300
GBM.14	$7.0\ 10^{-3} \pm 2.3\ 10^{-2}$ [7 10^{3}]		UM	$0.4 \pm 2.1\ 10^{-2}$	220
GBM.15	0.80 ± 0.05 [8 10^{5}]	++	UM	$1.33\ 10^{-3} \pm 0.11\ 10^{-3}$	>300
GBM.16	$5.0\ 10^{-2} \pm 0.11$ [5 10^{4}]	++	UM	$1.50\ 10^{-2} \pm 0.07\ 10^{-2}$	220
GBM.19	$2.3\ 10^{-3} \pm 0.6\ 10^{-3}$ [2.3 10^{3}]		UM	$1.9\ 10^{-2} \pm 0.7\ 10^{-3}$	>300
GBM.21	$3.0\ 10^{-3} \pm 0.9\ 10^{-4}$ [3 10^{3}]	++	UM	$6.8\ 10^{-2} \pm 0.8\ 10^{-2}$	>300
GBM.22	$1.8\ 10^{-2} \pm 1.0\ 10^{-4}$ [1.8 10^{4}]	++	UM	$7.3\ 10^{-3} \pm 1.6\ 10^{-3}$	>300
GBM.23	0.12 ± 0.05 [1.2 10^{5}]	++	UM		>300
GBM.24	$1.9\ 10^{-3} \pm 1.3\ 10^{-3}$ [1.9 10^{3}]		M	$1.5\ 10^{-2} \pm 2.1\ 10^{-2}$	>300
GBM.25	$2.0\ 10^{-6} \pm 0.3\ 10^{-6}$ [2]	nd	M	1.64 ± 0.11	180

*in brackets, relative expression level of MGMT, taking MGMT level in U87 cells as 1 (cf **Table 1**); nd, no detectable; M, methylated MGMT; UM, unmethylated MGMT.

found to be little affected by TMZ (IC_{50} > 300 μM), a dose-dependent inhibition of the proliferation of U87, U373, and U118 cells was observed, with IC_{50} of 50 μM, 110 μM, and 220 μM respectively. The effect of TMZ on cell proliferation was also examined in a panel of human tumoral glial cells established in primary culture from glioblastomas. Data reported in **Figure 1B** indicate that most of the primary cultures were unaffected by the presence of TMZ (150–300 μM). However, a dose-dependent inhibition of cell proliferation was observed in two primary cultures treated with TMZ (GBM.14 and GBM.16), with IC_{50} of 220 μM. The IC_{50} values of TMZ for each cell line and glioblastoma primary culture are listed in **Tables 1 and 2**, respectively.

In order to verify the mechanism through which TMZ affects cell glioma proliferation, its effect on cell cycle was further analyzed. In agreement with a number of reports, our data confirm that the growth-inhibitory effect of TMZ is associated with accumulation of cells into G_2/M cell cycle phases and concomitant decrease of cell number into G_0/G_1 (**Figs. S1 and S2**).[46-48] These results confirm that TMZ is able to affect glioma cell growth by blocking the transit beyond cell cycle phases.

Expression level of molecular markers in GBM and lower grade gliomas tissue samples

A number of genes are associated with tumor grade, progression and patient survival with GBM.[46,47,49,50] The expression of several genes of interest was measured in a panel of high (GBM) and lower grade gliomas. In parallel, several tumoral cell lines issued from GBM were included in the study. Data reported in **Figure 2** indicate that the expression of PDGFR-α, VEGFc, BCAN, MGMT, and Bcl_2:Bax ratio were not significantly different in GBMs, tumoral cell lines, and lower grade gliomas. On the contrary, TIMP1, YKL-40 (chitinase), and vimentin, described to be implicated in the malignancy of gliomas, appeared overexpressed in GBMs and tumoral cell lines when compared with lower grade gliomas.

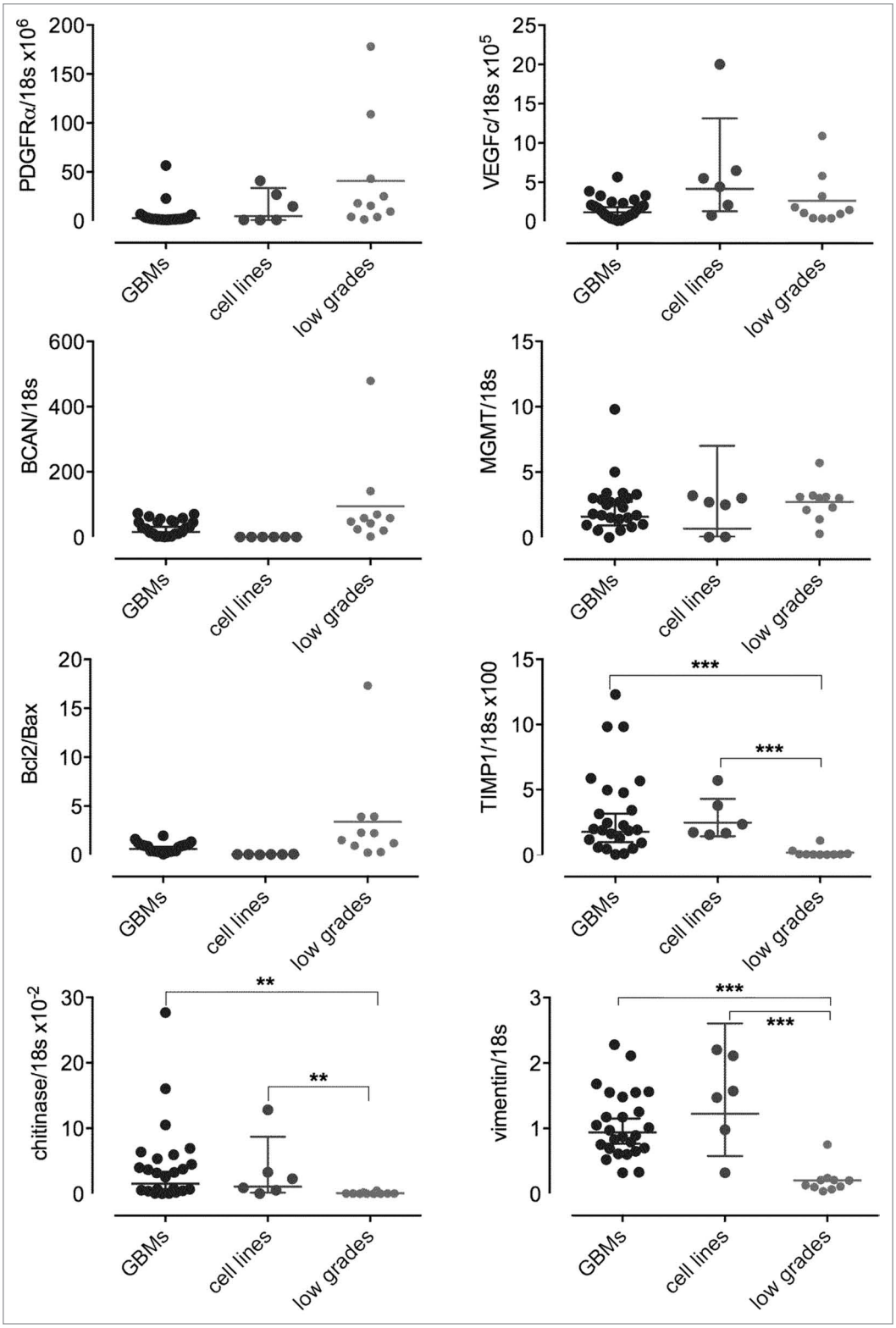

Figure 2. Relative expression of biomarkers in grade IV (GBMs) and grade II–III gliomas (low grade) and in tumoral glial cell lines. Expression levels were measured by quantitative RT-PCR and values were normalized with 18s housekeeping gene. For each data set, median value and 5% and 95% confidence intervals are reported. $^{**}P < 0.01$; $^{***}P < 0.001$

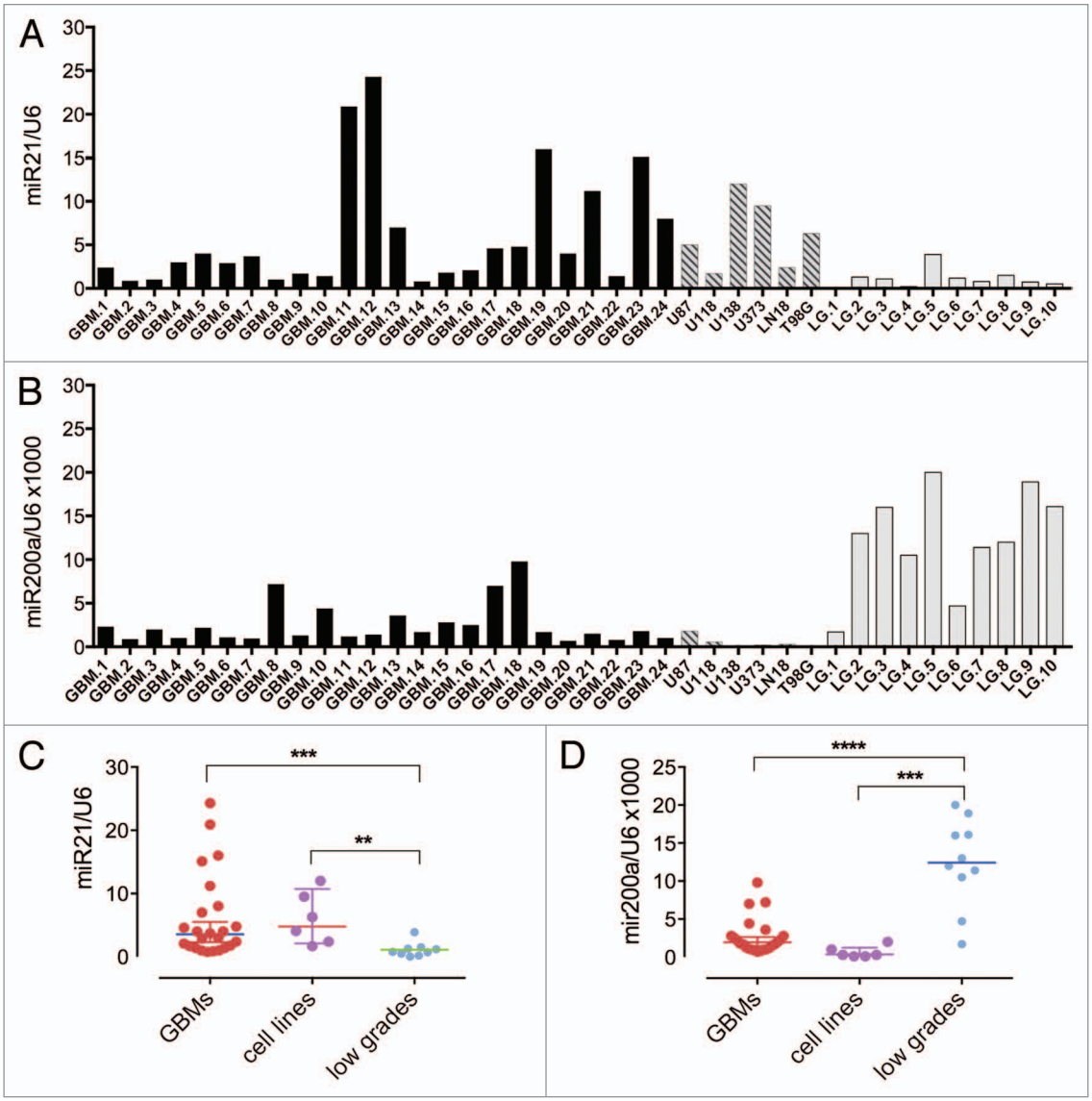

Figure 3. Relative expression of miR-21 (**A and C**) and miR200a (**B and D**) in grade IV (GBM) and grade II–III gliomas (low grade), and in tumoral glial cell lines. Expression levels were measured by quantitative RT-PCR and values were normalized with snU6 housekeeping gene. For each data set, median value and 5% and 95% confidence intervals are reported (**C and D**). $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

MiR200a and miR21 expression levels in GBM tissue and lower grade gliomas tissue samples

In order to determine whether miR21 and miR200a may be associated to brain tumor progression, the expression of both miRNAs was measured in lower grade (LG) and GBM samples, as well as in tumoral glial cell lines. The relative expression levels reported in **Figure 3** demonstrate that miR21 was significantly overexpressed in GBM compared with LG samples ($P < 0.001$). Similar results were observed when comparing miR21 expression in GBM-derived cell lines vs. LG samples ($P < 0.01$). Conversely, miR200a expression level was significantly lower in both GBM samples and glioma cell lines, compared with LG ($P < 0.0001$ and $P < 0.001$, respectively).

miR200a and mir21 affect TMZ-tumoral glial cell responsiveness

An increasing number of microRNAs are described to be involved in tumoral cell response to chemotherapy. To evaluate the potential role of miR200a and miR21 in tumoral glial cell responsiveness to TMZ, the expression of both miRNAs was first determined in a panel of glioblastoma cell lines (U87, U373, T98, and LN18) treated in the absence or in the presence of TMZ. Our data indicate that under our experimental conditions, TMZ was unable to affect the expression of miR200a and miR21 in all the cell lines as well as in several GBM primary cultures (GBM.10, GBM.11, GBM.14, and GBM.16) examined (not shown).

In a second time, miRNA transfection studies were performed to assess the potential involvement of miRNA, in particular miR200a, in TMZ-responsiveness. For this purpose, U87 and U373 cell lines were transfected with either the antagomir of miR21 (as-miR21) or the mimic pre-miR200a. Control cells were transfected with the respective control oligonucleotides. RT-PCR measurements performed 24 h and 48 h post-transfection demonstrated decreased expression of miR21 and overexpression of miR200a, respectively (**Figs. S3 and S4**). Whereas TMZ at the indicated concentrations had no significant effect on the proliferation of cells transfected with pre-miRNA control, the addition of TMZ in U87 and U373 cells overexpressing miR200a decreased cell growth to 60% of control (**Fig. 4**). As expected, similar results were obtained after cell transfection with the miR21 inhibitor. Furthermore, the effect of miRNA transfection on TMZ-responsiveness was verified in two GBM primary cultures, previously shown to display different growth response to TMZ. Thus, whereas the proliferation of GBM.16 primary culture was reduced in the presence of TMZ (IC_{50} = 220 μM), GBM.22 appeared to be TMZ-unresponsive (cf. **Fig. 1**). Data reported in **Figure 4** show that TMZ decreased the proliferation of GBM.16 overexpressing miR200a to 50% of control, *vs* 82% in cells transfected with pre-miRNA control. Likely, the reduction of miR21 expression in the same cells resulted in an increased TMZ growth inhibition. Conversely, neither miR200a overexpression nor miR21 inhibition could modify the response of GBM.22 primary culture to TMZ.

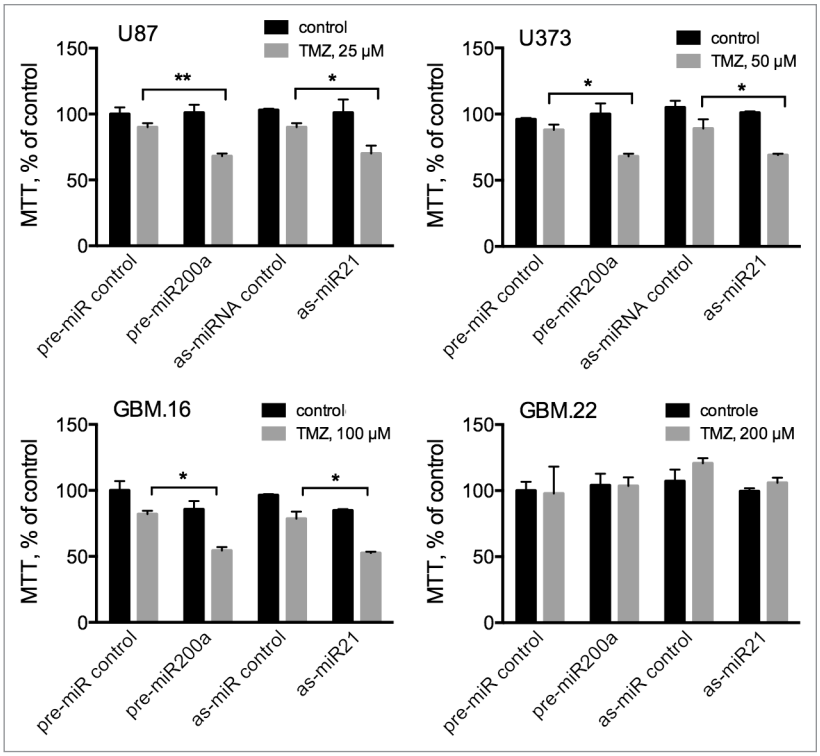

Figure 4. Effect of miR21 inhibition and miR200a overexpression on TMZ-responsiveness. Tumoral glial cell lines (U87, U373) and glioblastoma-derived primary cultures (GBM.16, GBM.22) were transfected with anti-sens miR21 (as-miR21) or mimic premature miR200a (pre-miR200a). Control cells were transfected with anti-sens miRNA control (as-miR control) or premature miRNA control (pre-miR control). Three days post-transfection, cells were treated for 3 d with the indicated concentrations of TMZ. Cell proliferation was then assessed by MTT metabolism. Values are expressed as % of the respective untreated control cells. Data are the mean ± SD of triplicate and are representative of 2 separate experiments.

miR200a expression is correlated with MGMT expression and TMZ glial cell responsiveness

Since miR200a and miR21 were shown to be involved in TMZ-responsiveness of a number of tumoral glial cells, data analysis was conducted to verify whether both miRNAs could be used to discriminate TMZ-responsive and -unresponsive GBMs. GBM primary cultures were then divided into two subgroups. The first one contained primary cultures that displayed growth inhibition in the presence of TMZ (IC_{50} = 200–250 μM). The second group included primary cultures that were unaffected by the presence of TMZ in our experimental conditions (IC_{50} > 300 μM). Data relative to the characterization of each primary culture used in this analysis are reported in **Table 2**.

As MGMT is largely admitted to play a crucial role in mediating the resistance to alkylaling agents such as TMZ, the correlation between MGMT expression and TMZ responsiveness was examined. Immunoblot analysis of MGMT in a panel of GBM primary cultures verified that MGMT expression was associated, in most of them, with the presence of MGMT protein (**Fig. 5**; **Table 2**). Data reported in **Figure 6A** confirm that MGMT expression levels were significantly higher in GBM primary

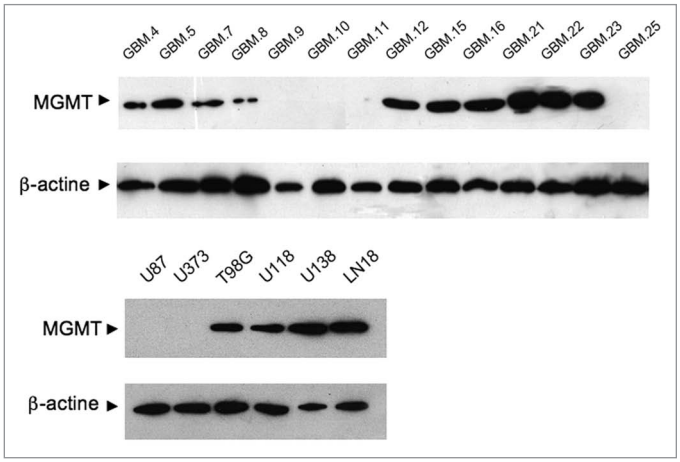

Figure 5. Expression of MGMT protein in GBM-derived primary cultures and tumoral glial cell lines. Cell lysates were prepared as described in Materials and Methods. After separation by electrophoresis and transfer on nitrocellulose membrane, MGMT protein was analyzed by immunobloting. In parallel, β-actin protein level in each sample was verified. Data are representative of two separate experiments.

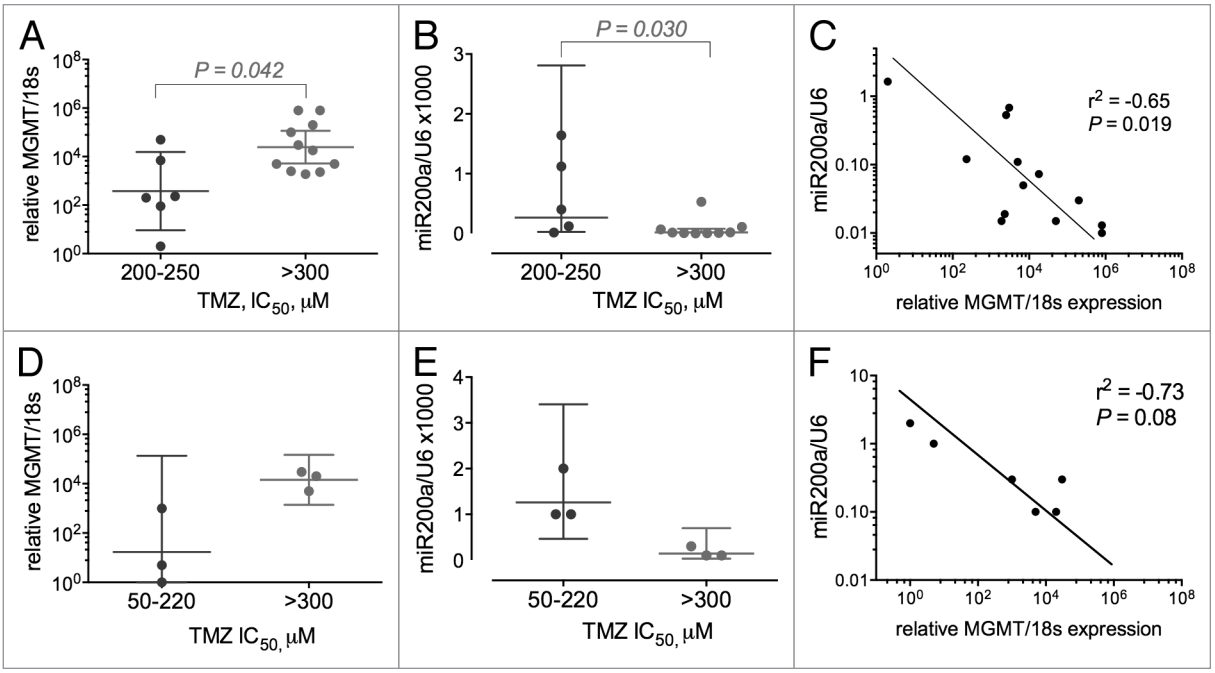

Figure 6. Correlation analysis between miR200a and MGMT expression levels and TMZ-responsiveness in GBM-derived cell primary cultures (**A–C**) and tumoral glial cell lines (**D–F**). Primary cultures and tumoral cell lines were subdivided into TMZ-responsive (IC_{50} = 200–250 μM and 50–220 μM, respectively) and -unresponsive (IC_{50} > 300 μM) groups. For each groups, median values and 5% and 95% confidence intervals for MGMT (**A and D**) and miR200a (**B and E**) are reported. Correlation analysis between MGMT and miR200a expression levels are presented (**C and F**).

cultures resistant to TMZ, compared with TMZ-responsive group (P = 0.042). Whereas both subgroups were shown to express similar levels of miR21 (not shown), miR200a expression was significantly increased in TMZ-responsive GBM cultures (P = 0.030) (**Fig. 6B**). Given these data, a potential correlation between miR200a and MGMT expressions was then examined. As expected, a negative correlation was observable between miR200a and MGMT (r^2 = –0.65, P = 0.019) (**Fig. 6C**).

The same analysis was conducted on tumoral cell lines. The TMZ-responsive (IC_{50} = 50–220 μM) and -unresponsive (IC_{50} > 300 μM) groups included U87, U373, U118, and LN18, T98G, U138 cell lines, respectively. Whereas the expression of MGMT tended to be higher in TMZ-resistant cells (**Fig. 6D**), miR200a expression level was augmented in TMZ-sensitive cell lines (**Fig. 6E**). However, these differences in expression levels were not significant, probably due to the small number of cell lines examined. Nevertheless, tendency to a negative correlation between miR200a and MGMT expressions was observable (r^2 = –0.73, P = 0.08) (**Fig. 6F**).

miRNA200a expression is regulated by MGMT activity

The correlation observed between MGMT and miR200a expression levels in tumoral glial cells suggests that molecular interactions between MGMT and miR200a might occur. In a first time, experiment was conducted to verify that the inhibition of MGMT could promote the growth inhibitory effect of TMZ in tumoral glial cells. The TMZ-unresponsive LN18 and T98G cell lines that were shown to express significant levels of MGMT (**Fig. 5**; **Table 1**) were treated with the MGMT inhibitor O^6-BG (50 μM) in the absence or in the presence of different concentrations of TMZ (100–300 μM). Our results demonstrate that TMZ at 300 μM was able to reduce T98G and LN18 cell proliferation to 66% and 60% of control respectively, when added in the presence of O^6-BG (**Fig. 7A**; **Fig. S5**). In parallel, the disappearance of detectable MGMT protein level in both cell lines under O^6-BG treatment was verified by western immunoblotting (**Fig. 7B**).

In order to determine whether the expression of miR200a and miR21 was dependent on MGMT activity, miRNA expression levels were then measured in cells treated with O^6-BG. The inhibition of MGMT activity allowed to an important increase of miR200a expression in LN18 cells, 24 h after the addition of 20–50 μM O^6-BG (**Fig. 8**). A moderate effect persisted 72 h after treatment with 40–50 μM O^6-BG (not shown). Similarly, miR200a expression level appeared significantly augmented 24 h after the addition of 20–50 μM O^6-BG in T98G cells. Conversely, O^6-BG was unable to significantly modify the expression of miR21 in both LN18 and T98G cell lines.

In a second step, potential regulation of MGMT expression by miRNA was examined. LN18 and T98G cells were then transfected with miR21 antagomir or pre-miR200a mimic, then MGMT expression was determined 48 h post-transfection. Results obtained demonstrate that neither reduction of miR21 nor overexpression of miR200a could modify the expression level of MGMT (not shown).

Discussion

It is now well established that particular miRNAs play a crucial role in gene regulation in health and disease. Notably, deregulation of a number of miRNAs has been demonstrated to be associated with cancer pathogenesis.[24] Furthermore, accumulating evidence has revealed that miRNAs may play a role in promoting tumoral cell response to chemotherapeutic agents, and a number of reports have proposed the use of miRNAs as potential predictive and prognostic factors in a variety of cancers including GBMs.[29,51-53] In this study, we demonstrate differential expression of miR21 and miR200a in grade IV (GBM) vs. lower grade (II–III) (LG) human brain tumors. Moreover, our data reveal for the first time a specific function for miR200a in promoting the response of tumoral gial cells to the alkylating agent TMZ.

MiR21 has been described to be significantly augmented in GBMs and to function as an oncogene that targets a variety of tumor suppressor genes.[27,30] However, the association of miR21 with grade in gliomas is less consistent. In agreement with the report by Hermansen et al.[29] we demonstrate herein that miR21 is overexpressed in GBM samples when compared with LGs. Likewise, cell lines derived from glioblastomas are shown to express higher miR21 levels than LGs, confirming that miR21 expression level is correlated with disease malignity, and indicating that it may discriminate grade IV from grade II–III gliomas. Contrary to miR21, the expression and function of miR200 family members in brain tumors are little documented. Saydam et al.[45] have shown that downregulation of miR200a results in the activation of the wnt/β-catenin signaling pathway and increases tumor growth in meningiomas. Also, miR200 family has been demonstrated to be involved in the suppression of EMT and self-renewal of GBM initiating cells by Erismodegib.[54] In agreement with these data, we were able to demonstrate for the first time that miR200a is downregulated in both GBMs and tumoral glial cell lines when compared with LGs, strongly suggesting that loss or reduction of miR200a expression occurs during GBM development and tumor progression.

Innate or acquired resistance to chemotherapeutic agents such as TMZ is a common fate in GBMs.[4-6] Consistent with this observation, we show that a few number of GBM primary cultures display growth inhibition in response to TMZ. Likewise, among 6 GBM-derived cell lines examined, 3 are shown to be TMZ-responsive. The presence of MGMT in tumoral glial cells has been shown to be responsible, at least in part, of TMZ resistance.[7,8,55] This is comforted by the association that we observe, between MGMT expression level and TMZ-growth inhibitory activity in both tumoral cell lines and GBM primary cultures. However, published data indicate that this correlation is far from being total, arguing for the involvement of

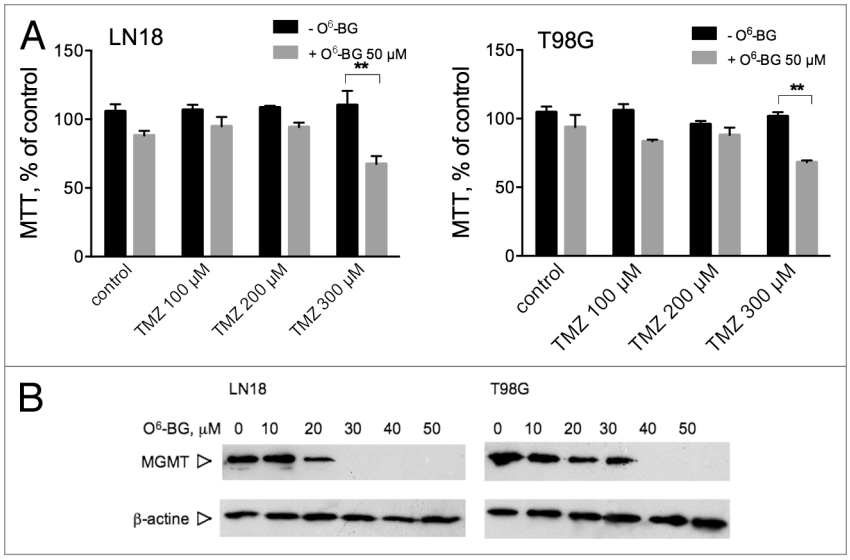

Figure 7. Effect of the MGMT inhibitor O⁶-BG on TMZ-responsiveness of tumoral glial cells. (**A**) T98G and LN18 cell lines were pre-treated with 50 µM O⁶-BG for 4 h. Cells were then treated in the absence or in the presence of TMZ at the indicated concentrations. Three days later, cell proliferation was assessed by MTT metabolism. Values are expressed as % of the respective untreated control cells. Data are the mean ± SD of triplicate and are representative of 3 separate experiments. $^{**}P < 0.001$. (**B**) the effect of different concentrations (10–50 µM) O⁶-BG on MGMT protein level was examined by immunobloting. In parallel, β-actin expression was verified for each sample. Data are representative of two separate experiments.

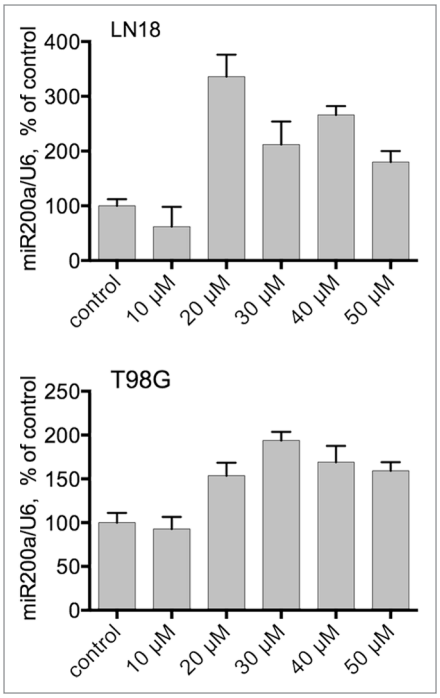

Figure 8. Effect of the MGMT inhibitor O⁶-BG on miR200a expression level in tumoral glial cells. T98G and LN18 cell lines were treated for 24 h with different concentrations (10–50 µM) of O⁶-BG. Expression levels of miR200a were measured by quantitative RT-PCR and values were normalized with snU6 housekeeping gene. Data are expressed as % of untreated control cells. Values are the mean ± SD of triplicate and are representative of 3 separate experiments.

MGMT-independent mechanisms in cell resistance to TMZ.[12] Thus, besides their involvement in tumor development and progression, a number of miRNAs have been shown to influence cell responsiveness to chemotherapies.[56] This is true for miR21, as increasing number of studies demonstrate that knockdown of miR21 is able to promote tumor growth inhibition triggered by chemotherapeutic agents.[57,58] Thus, miR21 has been described to mediate resistance to chemotherapy and ionizing radiation in GBMs through the inhibition of apoptotic signaling pathway.[59,60] In agreement with these data we confirm that depletion of miR21 increases TMZ-responsiveness of tumoral glial U87 and U373 cell lines. On contrary, TMZ is unable to modify the expression levels of miR21 in several tumoral glial cell lines and GBM primary cultures. These late data are in apparent contradiction with the report by Wong et al.[61] demonstrating that exposure of glioblastoma cells to TMZ elevated miR21 expression. However, miR21 augmentation was observed in glioblastoma cells that developed TMZ resistance after long-term, chronic administration of TMZ. In our study TMZ treatments did nor exceed 72 h, suggesting that regulation of miR21 by TMZ occurs via indirect mechanisms and requires profound phenotypic and/or genotypic changes.

Although the role of miR200a in tumoral cell responsiveness to therapy is less documented, it is obvious that the expression of miR200a in a variety of tumoral cells promotes cytotoxic activity of antitumoral drugs.[36,39,62] In agreement with these data, we show that the overexpression of miR200a in U87 and U373 cells increases the growth inhibitory activity of TMZ. Likely, increasing expression levels of miR200a promotes the response of GBM.16 primary culture to TMZ. Moreover, our results bring in evidence a correlation between miR200a expression level and in vitro growth response of GBM primary cultures to TMZ. Thus, primary cultures that display growth resistance to 300 μM TMZ are shown to express lower miR200a levels than TMZ-responsive GBMs. Although TMZ-responsive cell lines tended to express higher miR200a levels when compared with unresponsive cells, significant correlation cannot be evidenced, possibly due to the small number of cell lines examined. Interestingly, our data further indicate a negative correlation between miR200a and MGMT expression levels in GBM primary cultures. Similarly, miR200a tended to be negatively correlated with MGMT in tumoral cell lines, suggesting that reciprocal regulation between miR200a and MGMT occurs in glioblastoma cells. In agreement with these data, the inhibition of MGMT activity by O^6-BG in two different cell lines (LN18 and T98G), increases the expression of miR200a and partially reverses cell resistance to TMZ. However, modifying miR200a expression in LN18 and T98 cells did not affect MGMT level, suggesting unilateral regulation of miR200a by MGMT. The mechanism through which MGMT affects miR200a expression is still unknown. However, in deficient MGMT cells, O^6-MeG adducts produced under the action of a variety of mutagens may activate apoptotic pathway in a p53-dependent manner.[63,64] Furthermore, p53 has been shown to upregulate miR200 family members that in return suppress EMT by repressing the expression of ZEB1 and ZEB2 (SIP1).[65] Thus, we suggest that accumulation of O^6-MeG adducts in cells treated with O^6-BG might induce miR200a expression through a p53-dependent mechanism. Certainly, future investigations are needed to define the functional relationship between decrease of miR200a expression, MGMT activity and oncogenic features.

In conclusion, our present data confirm overexpression of miR21 and demonstrate downregulation of miR200a in high grade (IV) gliomas. Importantly, miR200a is shown to be able to promote chemotherapy responsiveness when overexpressed in glioblastoma cells. Further, the demonstration of the upregulation of miR200a by inhibition of MGMT activity provides new insight into the mechanisms responsible of glioma cell progression and chemo-resistance.

Materials and Methods

Materials

Cell culture medium and reagents, oligofectamine, random hexamers, MMLV, mirVanaTM kit, turbo DNA-free kit, miRNA assay kit, miRNA reverse transcription kit, and PCR TaqMan probe assays were provided by Invitrogen Life Technologies Inc. Temozolomide, O^6-benzylguanine (O^6-BG), thiazolyl blue tetrazolium blue (MTT), and collagenase were from Sigma-Aldrich. Enhanced chemiluminescent substrate was from GE Healthcare Life Sciences. Protease inhibitor cocktail and standard genomic DNA was furnished by Roche Applied Science. Tri-Reagent and DNA purification kit were from Euromedex and Promega, respectively. Anti-MGMT monoclonal antibody (clone MT3.1) and horseradish peroxidase-conjugated secondary antibody were from Epitomics and Santa Cruz Biotechnology (Tebu) respectively. Epitect bisulfite kit was provided by Qiagen.

Cell line culture

Human glioblastoma cell lines U87, U373, T98G, LN18, and U138 were obtained from the American Type Culture Collection. They were maintained in DMEM supplemented with 2 mM glutamine and 10% fetal bovine serum (FBS), except LN18 cells that were cultured in the presence of 5% FBS. Cells were cultured at 37 °C, under a humid atmosphere of 5% CO_2/95% air.

Primary cultures of tumor glial cells

Tumor samples classified as glioblastoma based on World Health Organization (WHO) criteria were obtained from patients undergoing surgical treatment at the Department of Neurosurgery, CHU Timone. Within 1–4 h after surgical removal, tumors were washed and dissected into small pieces (<1 mm^3) in RPMI-1640 medium supplemented with 2% FBS and 50 μg/mL penicillin/streptomycin, and disrupted mechanically. Tumoral glial cells were separated on a 30% continuous dextran gradient prepared in RPMI, washed in RPMI and subsequently dissociated in 10 μg/mL collagenase in RMPI. After 1 h at 37 °C, cells were washed before plating in T-75 cm^2 flasks. GBM primary cultures were maintained in DMEM/F12 supplemented with 10% FBS and 50 μg/mL penicillin/streptomycin, at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Cells were grown to confluence with medium change twice a week and used until passage 5. Glial cell cultures were characterized by the presence of GFAP and the absence of oligodendrocyte markers.

Patients

Molecular analysis was performed on 24 primary glioblastomas (GBMs) and 10 low grade brain tumors (LGs) resected at the Department of Neurosurgery, CHU Timone. All the tissue procurement protocols were approved by the relevant institutional committees (Aix-Marseille University) and were undertaken under informed consent of each patient or relatives. Diagnosis and grading of all tumors were certified by independent neuropathological examination. The histological diagnosis of GBM and LG was performed according to WHO guidelines. The mean age of the patients was 58.4 y (range = 27–81 y). Twenty-seven (79.4%) patients were males, and seven (20.6%) were females (**Table 3**). One viable sample from the tumors was stored at –80 °C for molecular analysis and one was fixed in buffered-formalin and embedded in paraffin. For each sample, at least 1 slice was stained with hematoxylin and eosin to control the percentage of tumor cells. Only samples containing at least 60% of tumor cells were retained for subsequent analysis.

Preparation of cell extracts and western immunoblotting

Cells were seeded at 15 000 cells/cm^2 in their regular culture medium. At pre-confluence, cells were rinsed with phosphate-buffered saline (PBS) and treated in fresh culture medium as mentioned. At the end of the treatment, cell monolayers were washed with ice-cold PBS and incubated in lysis buffer (25 mM TRIS-HCl pH 7.4, 150 mM NaCl, 2.5 mM EDTA, 50 mM sodium fluoride, 2 mM sodium orthovanadate, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonylfluoride) containing protease inhibitor cocktail as indicated by the manufacturer. Insoluble material was removed by centrifugation at 4 °C for 15 min at 20 000 × g. Cell extracts were resolved by SDS-PAGE and separated proteins were then electroblotted on a nitrocellulose membrane. Western immunobloting was performed as previously described.[66]

Quantitative real-time polymerase chain reaction (PCR)

RNA from tumor tissue samples was extracted using the mirVanaTM Kit according to the instructions of the manufacturer. Alternatively, total RNA from primary cultures of GBMs and tumoral glial cell lines was prepared with Tri-Reagent as specified by the manufacturer. In all cases, RNA samples were treated with DNase, using Turbo DNA-free kit. RNA purity was assessed by spectrophotometric measurement of the OD_{260}/OD_{280} and OD_{260}/OD_{230} ratios with acceptable values falling between 1.8 and 2.1. RNA integrity was verified by Bioanalyzer (Agilent Technologies). RNA (1 μg) was incubated at 70 °C for 10 min in the presence of 50 ng/mL of random hexamers. After cooling at 4 °C, RNA was reverse transcribed into cDNA in a reaction buffer containing 10 mM DTT, 1 mM deoxynucleotide triphosphates, 1.2 U/μL RNase inhibitor, and 10 U/μL of MMLV-RT. The reaction proceeded at 37 °C for 1.5 h, then at 70 °C for 10 min. SYBR Green real-time reverse transcription (RT)-PCR for the genes of interest was done using specific primers. For normalization, RT-PCR was performed using eukaryotic 18s rRNA endogenous control primers. Oligonucleotide sequences used in this study are reported in **Table 4**.

Table 3. Clinical and histopathological informations in 34 gliomas analyzed

ID	Age	M/F	Pathology	Treatment
GBM.1	72	H	GBM	STUPP
GBM.2	77	H	GBM	BCNU+TMZ
GBM.3	81	H	GBM	STUPP
GBM.4	58	H	GBM	STUPP
GBM.5	59	H	GBM	STUPP
GBM.6	65	H	GBM	STUPP
GBM.7	58	H	GBM	STUPP
GBM.8	76	F	GBM	BCNU+TMZ
GBM.9	42	F	GBM	STUPP
GBM.10	69	H	GBM	STUPP
GBM.11	65	H	GBM	STUPP
GBM.12	56	H	GBM	STUPP
GBM.13	53	H	GBM	STUPP
GBM.14	69	H	GBM	STUPP
GBM.15	68	H	GBM	STUPP
GBM.16	50	F	GBM	STUPP
GBM.17	80	H	GBM	BCNU+TMZ
GBM.18	65	H	GBM	STUPP
GBM.19	58	H	GBM	STUPP
GBM.20	59	H	GBM	STUPP
GBM.21	58	H	GBM	STUPP
GBM.22	42	F	GBM	STUPP
GBM.23	65	H	GBM	STUPP
GBM.24	69	H	GBM	STUPP
ODG.25	27	H	oligodendroglioma	-
AO.26	53	F	anaplastic oligodendroglioma	TMZ
AA.27		F	anaplastic astrocytoma	-
OA.28	45	H	oligoastrocytoma	-
OA.29	49	F	oligoastrocytoma	TMZ
AA.30	43	H	anaplastic astrocytoma	RT
AA.31	64	H	anaplastic astrocytoma	VCR+RT
ODG.32	52	H	oligodendroglioma	RT
ODG.33	40	H	oligodendroglioma	-
ODG.34	41	H	oligodendroglioma	RT

RT, radiation therapy; TMZ, temozolomide; BCNU, bis-chloroethylnitrosourea; VCR, vincristine; STUPP, radiotherapy plus concomitant and adjuvant temozolomide.

Specific quantitative real-time PCR experiments for microRNAs were performed using TaqMan MicroRNA assay. A total of 40 ng of RNA was used to reverse transcribe specific miRNA of interest into cDNA using the TaqMan® miRNA reverse transcription kit. The reverse transcription primers for miR21 and

Table 4. Sequences of forward and reverse primers used to quantify human mRNAs

Gene	Primers sequences
YKL-40	Forward primer 5′-GGACCACAGG CCATCACAGT-3′
	Reverse primer 5′-CCAGCCTCAA CATGTACCCC-3′
Vimentin	Forward primer 5′-AAAGTGTGGC TGCCAAGAAC-3′
	Reverse primer 5′-AGCCTCAGAG AGGTCAGCAA-3′
MGMT	Forward primer 5′-GGGTCTGCAC GAAATAAAGC-3′
	Reverse primer 5′-CTCCGGACCT CCGAGAAC-3′
PDGFRα	Forward primer 5′-GGACTTACCC TGGAGAAGTG AA-3′
	Reverse primer 5′-CCAATTTGAT GGATGGGACT-3′
TIMP1	Forward primer 5′-CTGTTGTTGC TGTGGCTGAT-3′
	Reverse primer 5′-AACTTGGCCC TGATGACG-3′
Bcl2α	Forward primer 5′-GCACCTGCAC ACCTGGAT-3′
	Reverse primer 5′-AGGGCCAAAC TGAGCAGA-3′
Baxα	Forward primer 5′-ATGTTTTCTG ACGGCAACTT C-3′
	Reverse primer 5′-ATCAGTTCCG GCACCTTG-3′
18s	Forward primer 5′-CTACCACATC CAAGGAAGGC A-3′
	Reverse primer 5′-TTTTTCGTCA CTACCTCCCC G-3′
BCAN	Qantitect Primer Assay: Hs_BCAN_1_SG (Qiagen)

miR200a are hairpin primers specific for the mature miRNAs and do not bind to the miRNA precursor forms. Reverse transcription was followed by real-time PCR using miRNAs specific TaqMan® probe assays (miR21-5p, ID 000397; miR200a-3p, ID 002274; control snU6, ID 001973) on a Roche LightCycler 480. Expression data were normalized according to the expression of snU6. The relative mRNA or miRNA levels were calculated using the comparative Ct method (ΔΔCt).

MGMT promoter methylation analysis

Genomic DNA was prepared as described by the manufacturer then converted by using the EpiTect bisulfite kit. Methylation analysis was performed using MethyLight Technique as described previously.[67] Briefly, real-time, fluorescence-based polymerase chain reaction was performed using the Light Cycler 480 (Roche Diagnostics). Bisulfite-converted genomic DNA was amplified using a set of primers and a fluorescent dye-labeled oligonucleotide probe, resulting in a semiquantitative methylation analysis. For the CpG islands of the 15 investigated gene regions (GENE), the primers and probes were designed specifically for methylated DNA. All primer and probe sequences have been published elsewhere, and the performance of the technique has been validated.[67,68] The differences in amounts of input genomic DNA were normalized by the collagen Type II, α1 gene (Col2A1). SssI-treated genomic DNA was used as a standard.

Cell growth assay

Cells were plated in 24 well-plates (10000–20000 cells/well). Two days later, culture medium was renewed and cells received TMZ for 3 d, at the concentrations indicated. When O^6-BG treatments were performed, cells were pre-treated with 50 μM O^6-BG for 4 h before the addition of TMZ. Control cells were treated in the presence of DMSO diluant. At the end of the treatment, cell proliferation was assessed with MTT. Briefly, 0.1 volume of MTT (5 mg/mL) was added in culture medium. After incubation at 37 °C for 1 h-2 h, culture medium was discarded and cells containing MTT formazan crystals were lysed in isopropanol. Optical density was measured at 570 nm, with background subtraction at 690 nm.

Cell transfection

The effect of miR21 and miR200a on chemosensitivity was evaluated by transfecting tumoral glial cells with miR200a precursor (pre-miR-200a) or antisense miR21 (anti-miR21) oligonucleotides (assay ID, PM10991 and AM10206, respectively), at 35 nM and 100 nM final concentrations, respectively. Cells were plated at 60000 cells per well in 12-well culture plates. Transfection was performed 24 h later in serum-free medium, using Oligofectamine transfection reagent and following the instructions of the manufacturers. In parallel, control cells were transfected with pre-miRNA or anti-miRNA negative controls. After 24 h, the medium was replaced with fresh medium containing 10% FBS. To evaluate the effects of the transfection on cell proliferation, cells were allowed to grow for additional 72 h in the absence or in the presence of TMZ as indicated. The expression of MGMT was evaluated 48 h post-transfection.

Statistical analysis

The Graph Prism version 6.0 software was used to generate statistical data. Statistical analysis of differences between miRNA levels in glioblastomas and lower grade brain tumors were evaluated using the non-parametric Mann–Whitney U-test between two groups. Correlations were calculated using Spearman correlation test. Significance was accepted at $P < 0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/cbt/article/28920/

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